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We had previously found that breast cancer cell expression of the transferrin receptor (TfR) and/or ability to proliferate in response to transferrin (Tf) correlated with the metastatic capabilities of the cells. We desired to elevate or lower breast cancer cell TfR expression using various techniques, and to assess as to whether or not these changes affected metastatic capability. The human MDA231 mammary adenocarcinoma cell line was transfected with a plasmid carrying the cDNA for human TfR in the sense orientation. High TfR expressors (MDA231/TfR) were selected by FACS, and were shown to express 6-12 times more TfR than did vector transfected control cells (MDA231/Neo). MDA231/TfR cells were observed to be able to proliferate in serum-free media via a non-Tf dependent mechanism whereas MDA231/Neo could not. When injected into the mammary fat pad of nude mice, both lines produced 1° tumors, however, MDA231/TfR cells formed frequent intra-peritoneal and lung micrometastases whereas MDA231/Neo cells formed only occasional lung micrometastases. Increasing cell surface TfR on the MDA231 cell line promoted cell serum-free growth and metastatic ability.

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Philip Cavanaugh 10/5/96
PI - Signature Date

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INTRODUCTION

Subject

The subject of this work is aimed at better understanding of breast cancer metastasis. Specifically, it proposes to explore as to whether or not breast cancer cell expression of the transferrin receptor (TfR) or proliferative response to transferrin (Tf) is related to increased metastatic behavior. The proposal outlined a number of techniques whereby breast cancer cell TfR would be increased or decreased by transfection; or where proliferative response to Tf would be manipulated using cell culture selection methods. In addition, exploration into tumor cell production of Tf and cytokine mediated changes into tumor cell TfR expression would be made.

Purpose

Demonstration of involvement of the Tf/TfR system in breast cancer metastasis would aid in basic understanding of the metastatic process. Possibly, new prognostic tools would evolve from the study. New functions attributed to the Tf/TfR system would enhance understanding of cell biology in general as well as to add to the knowledge of metastatic cell behavior.

To transfect low metastatic mammary tumor cells with the sense gene for the transferrin receptor and to determine the affect of increased expression on tumor cell proliferative response to Tf and on metastatic capability.

Since a major body of work has been accomplished by others using molecular biology techniques and transfection technology to produce and examine the affect of increased expression of a protein on cell function, the use of these types of procedures was a natural consideration in the study of TfR expression on tumor cell behavior.

To transfect highly metastatic cells with the antisense gene for the transferrin receptor and to determine the affect of decreased TfR expression on tumor cell proliferative response to Tf and on metastatic capability.

The expression of many cellular factors may be responsible for high metastatic capability. Increasing the expression of one of these in a low metastatic cell hypothetically deficient in a number of responsible factors, would therefore have no effect. However, if a highly metastatic cell is depleted of one factor necessary for metastatic function, then the chances of observing a decrease in metastatic behavior would be greater. Thus, the transfection of cells with a plasmid which would produce constitutive expression of an antisense TfR message is a logical choice of experimentation.

To isolate high or low TfR expressing tumor cells from a mixed population by using selection or sorting techniques. To determine the metastatic capability of the selected cells.

Many *in vitro* selection techniques have been used to obtain populations of tumor cells which are more or less metastatic than the parental population. As these types of experiments can also make strong statements about cellular requirements for metastatic behavior. Initially, cells will be selected for enhanced response to Tf using an *in vitro* selection process, and cells expressing high or low levels of TfR will be sorted using FACS.

Scope

One rat model for breast cancer existed in the laboratory at the genesis of these experiments: the rat 13762NF mammary adenocarcinoma. Cultured 13762NF cell lines of low and high metastatic capability (in syngeneic fisher 344 rats) were available. Two human breast cancer models were also in place: the MDA series and the MCF7 series. The human lines form primary breast cancers and varying degrees of metastatic lesions in nude mice. These lines were used as subjects for transfection and other

TfR manipulation studies. Any changes in the metastatic behavior of the lines in their host animals was used as an indicator of a true TfR induced change in metastatic capability

Background

Breast cancer deaths often result from complications arising from distant metastases, and not from symptoms arising from the primary tumor (1). Therefore, determining which tumor cell properties contribute to increased metastatic capability is necessary to resolve the breast cancer problem. Adhesion factors or growth factors present at the preferred site for metastasis and the ability of tumor cells to interact with those agents is thought to be a major mechanism involved in the formation of metastatic tumors (2).

Previous studies by us explored the possibility that tumor cell metastasis to the lungs was in part enhanced by the ability of the tumor cells to proliferate in response to factors specifically encountered in the lung environment. Cells of high and low lung-metastasizing capability from the rat 13762NF mammary adenocarcinoma were examined for their ability to proliferate in media conditioned by viable rat lung fragments. A response was seen for the high lung-metastasizing cells only. The major lung-derived lung metastasizing tumor cell mitogen was purified from the conditioned media and was found to be the iron transport protein transferrin (Tf; 3-5). Subsequent studies by us showed a correlation between tumor cell response to transferrin and metastatic capability in 5 of 6 animal and human tumor model systems (6,7).

The transferrin receptor (TfR), a $M_r \sim 180,000$ homodimeric integral membrane glycoprotein (8), binds two iron saturated Tf molecules and is responsible for the delivery of iron into cells either through internalization of Fe-Tf (9) or by activation of a plasma membrane associated NADH dependent oxidoreductase which mediates the trans-plasma membrane transport of iron from Tf (10). Rapidly dividing cells, including a variety of tumor cells (8,11,12), usually express high levels of TfR. Other investigators have shown that tumor cell expression of TfR, as determined by histochemical analysis, has been shown to correlate with tumor grade or stage and/or progression and metastasis in human breast carcinomas (13), human bladder transitional cell carcinomas (14), and in human malignant melanoma (15). High levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (16). Transferrin receptor expression was seen to be increased in a human melanoma line selected for metastatic capability in nude mice, when compared to the poorly metastatic parental population (17). Transferrin has been shown to be the major mitogen in bone marrow for bone marrow metastasizing human prostatic carcinoma cells (18), and proliferative response to Tf has been shown to be associated with malignant progression in a series of murine B16 melanoma sublines (19). Also, murine B16 melanoma cells selected to metastasize to the liver display an enhanced response towards hepatocyte localized transferrin (20).

Transferrin's proliferative effect on cells is thought to be due primarily to its ability to transport iron into the cell, thus maintaining the activity of key enzymes required for proliferation. One major site of iron need in rapidly dividing cells is the enzyme ribonucleotide reductase (8). However, some studies have suggested that iron transport alone cannot explain transferrin's growth stimulating activity (21-23). Also, iron delivered by Tf has been shown to mediate processes that soluble iron can not (24), and Non-iron internalization dependent events caused by Tf (or anti-TfR) binding to TfR have been described (25-29). The cells where these events are best studied and partially characterized are T-lymphocytes, where various lymphokine secretion and protein phosphorylation events occur upon anti-TfR binding to TfR (25,28,29). These events require TfR association with CD3- ζ and possibly involve lymphocyte-associated kinases ZAP-70, Fyn, and Lck (25).

The observation of a cellular property associated with and perhaps thought to be responsible for metastatic activity has frequently led metastasis researchers to pursue *in vitro* selection techniques

whereby tumor cells possessing high or low levels of a metastasis-associated marker or activity could be isolated. The proposed influence on metastatic behavior that the selected property played could then be determined by assessing the metastatic capability of the selected cells *in vivo* (30-34). These types of experiments have resulted in the identification of a number of tumor cell phenotypes thought to be associated with the ability to form metastases (30-34).

The transfection of low or high metastatic tumor cells with sense or antisense genes encoding factors thought to affect metastatic behavior has also been a frequently performed procedure. Thus, the molecular approach to elevating or depressing the expression of a given tumor cell protein could be performed to determine the affect of that expression on metastatic capacity. A number of studies have been performed whereby the sense expression of a protein has been shown to influence metastatic capability (35-37). Likewise, similar types of studies have demonstrated that the depletion of a factor by antisense expression of its message has altered the invasive capability of malignant cells (38).

Based on our previous observations, we thought that the determination of a possible involvement of TfR in breast cancer metastasis could be explored by breast cancer cell selection or transfection techniques. The transfection of cells with the sense gene for TfR has been accomplished by others (39,40). The bulk of these studies were performed using normal cells, the purpose to gain basic information on the functioning of TfR. One report has been published whereby the sense or antisense TfR gene has been transfected into tumor cells (41). However, no conclusions were made about the alteration of any tumor cell behaviors caused by the transfection process.

BODY:

MDA231 sense TfR transfection:

The major body of work performed in the past year on this grant was with the TfR transfected MDA 231 human mammary adenocarcinoma cell line. Spontaneous metastasis assay results were performed using the line and indicated that the increased TfR expression did enhance the metastatic capability of the cells. Also, an increase in the ability of the cells to proliferate in serum-deficient culture media was seen. These results have been presented at two meetings: the American Association of Cancer Research meeting (Washington DC; April 20-24; 42), and at the Metastasis Research Society congress (Gent, Belgium; September 5-11; 43)

Plasmid construction:

A sense TfR encoding construct was made by ligating the TfR coding region from pcDTR1 into the multiple cloning site of pcDNA1Neo (Invitrogen, San Diego, CA). The resulting plasmid was named pcDNA1/Neo/TfR (Figure 1). Vector only transfected cells were transfected with the pcDNA1Neo plasmid only. All lines were transfected using Lipofectamine and were selected using 400 µg/ml G418, and maintained in media containing 400 µg/ml G418.

Cells:

The MDA231 (also named MDA MB 231) line was grown in DME/F12 (1:1) media containing 5% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ environment. Cells were routinely checked for mycoplasma contamination.

Transfection:

Cells were grown to 30-50 % confluence in six-well plates. Cells were placed in serum-free media and transfected for eight hours with 0.2 - 0.5 µg plasmid DNA per well in 2.5 % Lipofectamine (Gibco, Grand Island, NY) as directed by the supplier. Cells were placed in conventional growth media for 24h, then in media containing 400 µg/ml geneticin (G418; Gibco). Surviving cells were passaged as usual and maintained in DME/F12 media containing 5% FBS and 400 µg/ml G418.

Cell Growth Assays:

Cells were removed from stock plates and seeded in 100 µL α-MEM containing 1% FBS at a density of 2,000 cells/well in 96 well plates. One day later, cells were washed with three changes of α-MEM and the medium was changed to 100 µL α-MEM containing 0.0% FBS. On ensuing days, cells in select wells were trypsinized and cells were quantitated using a model ZM Coulter Counter.

Immunofluorescent Detection of Cell Surface TfR

All washes and antibody incubations were performed using 25 mM HEPES (pH 7.5) buffered Dulbecco's-modified minimal essential medium containing 1% v/v liquid gelatin (DMEM-LG). Cells grown on LabTek (Nunc, Naperville, IL) slides were washed 3 times with DMEM-LG and equilibrated to 4° C. Phycoerythrin (PE) conjugated mouse IgG₁ or PE-conjugated anti-human TfR (clone T56/14, Biotest International, Kennebunkport, ME) was added to the chambers to a final dilution of 1:200. Cells were incubated for 1 h at 4°C, were washed three times with DMEM-LG, and examined for fluorescence using a Nikon Diaphot phase contrast microscope.

Rhodamine-Tf Uptake:

Cells were grown on multi-chambered LabTek slides to 40-50 % confluence. Cells were washed twice for 1 h each in α -MEM only. Media was changed to DMEM-LG and rhodamine conjugated human Tf was added to a final concentration of 0.5 μ g/ml. Negative control wells also received un-conjugated Tf at a concentration of 20 μ g/ml. Two hours later, wells were washed three times with DMEM-LG, equilibrated to 4°C, and cells were examined for fluorescence.

FACS analyses and sorting:

All washes and antibody incubations were performed using 25 mM HEPES (pH 7.5) buffered Dulbecco's-modified minimal essential medium containing 1% v/v liquid gelatin (DMEM-LG). Cells were removed from culture plates using 0.25% trypsin, 2 mM EDTA (in Ca^{++} , Mg^{++} -free PBS). As soon as detachment was complete, FBS was added to 1% (v/v), to neutralize the trypsin. Cells were washed twice by centrifugation and re-suspension in DMEM-LG, counted, cell density was adjusted to 1×10^6 /ml, and the cell suspensions were equilibrated to 4°C. Phycoerythrin (PE) conjugated mouse IgG₁ or PE conjugated anti-human TfR was added to the suspensions to a final dilution of 1:200. Suspensions were incubated at 4°C for 1h, washed three times with DMEM-LG, and analyzed for fluorescence using a Becton-Dickinson FACStar instrument.

Cell synchronization:

Cells were grown to 30-40% confluence. Growth media was replaced with that containing 3 μ M aphidicolin (diluted from a 1 mg/ml stock solution in DMSO) for 12 h. Media was replaced with aphidicolin-free media for 12h, and then with aphidicolin containing media for 12 h. Cells were removed with trypsin and analyzed for TfR expression as outlined in the FACS analysis section.

Spontaneous Metastasis Assays:

Cultured cells were removed from culture plates with Ca^{++} , Mg^{++} - free Hank's basic salt solution (CMFH) containing 1 mM EDTA and 0.25% trypsin. Cells were washed 3 times with CMFH, cell density was measured using a model ZM Coulter Counter and adjusted to 5×10^6 /ml with CMFH. Cells were kept at room temperature and were injected in a 0.1 ml volume into the left thoracic mammary fat pad of Metofane anesthetized 6-8 week old female nude mice. Six weeks later, animals were killed with an overdose of Metofane and were examined for the presence of metastatic lesions.

Affinity Isolation of TfR using Immobilized Tf:

Tf-Agarose preparation:

Cyanogen bromide-activated agarose was washed with 10 volumes of 1 mM HCl and equilibrated in a 0.1 M NaHCO_3 , 0.5 M NaCl, pH 8.3 coupling buffer. Apo-Tf was added to the agarose in coupling buffer at a ratio of 10 mg protein /ml packed gel. The mixture was incubated at 4° C overnight in an end over end mixer. The gel was treated with 1 M ethanolamine, pH 8.0 for 2 h at 25° C and was washed with coupling buffer, then with 0.1 M acetate, pH 4.0 containing 0.5 M NaCl, and again with coupling buffer. The immobilized Tf was iron saturated by exposure to 1 mg/ml ferric ammonium citrate in 0.1 M NH_4HCO_3 . The gel was washed with 10 volumes of PBS containing 0.5% v/v Triton X-100.

Assay:

To reduce inherent bound Tf, cells were incubated in two changes of α -MEM only (2h each) prior to the analysis. Cells (70-80% confluent in 100 mm dishes) were then washed with 3 times with 5 ml PBS at 4°C, and 3 ml ice cold PBS containing 1.0 mg NHS-LC-biotin was added. Dishes were incubated at 4°C for 90 min while shaking, cells were washed 5 times with 5 ml PBS and lysed in 3 ml (per dish) PBS containing 2.0% Triton X-100, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM iodoacetamide, 10 mg/ml leupeptin, and 10 mg/ml aprotinin, at 4°C for 2 h. The lysate was centrifuged at 13,000 X g for 10 min. Lysate supernatant protein was determined using the detergent compatible BCA (Pierce, Rockford, IL) assay. The lysate supernatant (1-2 mg total protein) was combined with an excess of Tf-agarose (0.4 ml packed gel) and incubated for 2 h at 37°C. The gel was harvested by centrifugation at 2,000 x g, and washed 3 times by suspension in PBS containing 2% Triton X-100, and repeated centrifugation. Bound cell lysate proteins were released by exposure of the gel to 0.4 ml non-reducing SDS-PAGE sample treatment solution at 95°C for 10 min. The samples were separated on a 7.5 % SDS-PAGE gel according to Laemmli (28) and blotted onto immobilon (Millipore, Bedford, MA) membranes using a 10 mM CAPS, pH 10.0 transfer buffer. The membrane was blocked for 2 h at 25 °C with PBS containing 10% nonfat dry milk and 0.5% Tween 20 and was incubated with blocking solution containing streptavidin-HRP (1:2000) for 1 h at 25°C. The membrane was washed 4 times with 40 ml PBS containing 0.5% Tween 20. HRP-ECL substrate (DuPont, Wilmington, DE) was applied, light emitting bands were detected by autoradiography and were quantitated using a Hoeffer (San Francisco, CA) model GS300 scanning densitometer.

Scatchard analysis of cell surface TfR:**¹²⁵I-Tf preparation:**

To 1 ml of a 1 mg/ml solution of human holo-Tf (in 25 mM HEPES, pH 7.5) was added 0.5 mCi of Na¹²⁵I (ICN, Cleveland, OH) and 10 μ L of a 10 mg/ml solution of chloramine-T. This mixture was incubated at 25°C for 1 h and 10 μ L of a 20 mg/ml of sodium bisulfite solution was added. The sample was passed through a PD-10 column (Pharmacia, Uppsala, Sweden) which was equilibrated and run with PBS. One ml fractions were collected, and aliquots of each fraction were examined for radioactivity using a gamma counter. Radioactive fractions corresponding to the void volume were pooled. Specific activity was \approx 300,000 cpm/ μ g protein

Assay:

Cells were grown in 12-well plates to 70-80% confluency. To reduce inherent bound Tf, cells were incubated in two changes of α -MEM only (2 h each) prior to the analysis. Cells were washed twice with PBS, and 1 ml PBS containing 1% v/v liquid gelatin (Sigma, as a non-specific blocking agent) was added. The plates were equilibrated to 4°C and increasing levels of ¹²⁵I-Tf were added in quintuplicate to wells. Immediately prior to the addition of ¹²⁵I-Tf, two wells of each dose received a 50-times excess of unlabeled Tf. Wells were incubated for 2 h at 4°C, media containing unbound counts was saved, and wells were washed 5 times with PBS. Cells were lysed with PBS containing 2% Triton X-100, and lysate and unbound counts were determined using a Packard A5550 gamma counter.

¹²⁵I-Tf Uptake and Release:

Cells were grown to 20-40% confluence in six well plates. To reduce cell associated Tf originating from the growth media, cells were washed three times for 1h each with α -MEM only. Cells were then incubated for 8 h in DMEM-LG containing 0.5 μ g/ml ¹²⁵I-Tf. Cells were washed extensively and cells from three wells of both lines were lysed and radioactivity determined; remaining wells received

DME/F12 only. On ensuing days, three wells from both lines were analyzed: radioactivity was determined in removed media and in cell lysates, so that secreted and cell-associated Tf could be determined.

Western blot detection of Tf:

Initial treatment:

Stock cultures were washed 3 times for 1h each in alpha-MEM only. Cells were trypsinized and initially plated for 12 h in media containing 1% human serum in place of FBS. The cells were washed extensively with serum-free media, placed in serum-free media, and lysates were obtained at that time (day 1). On following days, lysates and media aliquots from like-plated and treated wells were obtained (days 2 and 3).

Detection:

Cells were lysed from plates in RIPA buffer (PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 0.5 U/ml aprotinin). Cells were incubated in RIPA for 1h at 4°C and centrifuged at 3,000 X g to remove particulates. Sample protein determination was performed using the BCA assay. To equal protein samples was added 1/3 volume of 4X SDS-PAGE treatment solution, samples were separated by SDS-PAGE, and blotted onto nitrocellulose membranes. Membranes were blocked with TBS (10 mM Tris, 0.15 M NaCl, pH 8.0) containing 0.1 % tween-20 and 5% non-fat dry milk. Membranes were incubated with a 1:1000 dilution of goat anti-human Tf (Sigma, St. Louis, MO) in block, were washed in TBS-tween and incubated for 2h at 25°C with a 1:1000 dilution of HRP-conjugated anti-goat-IgG (Sigma) in blocking solution. Membranes were washed with TBS-tween, ECL HRP substrate was applied, and light emitting bands were detected by autoradiography.

RESULTS:

The construction of a eucaryotic expression plasmid containing the cDNA coding for human TfR in the sense orientation driven by the CMV promoter using conventional cloning techniques was successfully performed. A schematic of the construct is shown in figure 1.

MDA231 cells were transfected with pcDNA1Neo or pcDNA1Neo/TfR with excellent efficiency. With both plasmids, enough G418 resistant cells were obtained per well that wells eventually became confluent. So, uncloned populations of transfected cells were trypsinized and expanded as usual, but in media containing G418.

Immunofluorescent analysis revealed that MDA231/Neo cells expressed undetectable levels of TfR as the mean level of fluorescence was identical to the isotype stained preparation (figure 2). Approximately 50% of the MDA231/TfR cells exhibited greater than background staining, indicating expression of the TfR protein (figure 2C). The top 5% of TfR expressing cells were sorted out and re-analyzed, indicating successful sorting (figure 2D).

The initial sorted population was expanded in culture and re-sorted, with the top 2% of TfR expressors being harvested. These were expanded and like sorted three more times. Immunofluorescent analysis of TfR expression on the cells from the first and fourth sort indicated that the sorting process increased TfR expression, but to a relatively small degree (figure 3). Representative photographs of immunofluorescent analysis of TfR expression on MDA231/Neo and MDA231/TfR FACS4 cells is shown in figure 4. These cells are stained unfixed at 4°C and are thus in the native state: they display a similar magnitude of staining difference as do those prepared for FACS. This indicates that trypsinization and removal from the culture surface (to which the latter cells have been exposed) has a minimal effect on immunofluorescent anti-TfR signal.

Heterogeneity in TfR expression was seen when any one MDA231/TfR population was analyzed (figures 1 and 2). Expression of TfR can vary with the cell cycle (8). To determine if cells should be synchronized prior to sorting, MDA231/TfR cells from the second sort were treated with aphidocolin. Cells were synchronized to the haploid state (G1 to S boundary) as shown in figure 5. Analysis of TfR expression on synchronized and unsynchronized populations was identical (figure 6) indicating other causes of heterogeneity and no need for synchronization prior to sorting.

Unlike results obtained with immunofluorescent analysis, analysis of cell surface TfR by affinity isolation did indicate that MDA231/Neo cells did possess some TfR protein. Successful transfection was apparent, as expression of TfR on MDA231/TfR cells was 6-7 fold higher (figure 7). The identity of the non-TfR bands seen which bind to Tf-agarose is unknown. The one at Mr = 70,000 may be residual cell associated Tf, however, results obtained from other experiments don't support this (data not shown).

Determination of cell surface TfR on both lines by Scatchard analysis revealed specific saturable Tf binding by both lines by one class of receptors. Here, as with affinity isolation results, MDA231/Neo cells were seen to possess some TfR. This assay indicated that MDA231/TfR cells expressed 6-7 fold more TfR than did the Neo cells (figure 8). It is not sure whether the differences in K_d between the two lines is significant. Also, insufficient amounts of ¹²⁵I-Tf were added to the TfR cells to obtain data near the x-axis, as such, extensive extrapolation was used to determine the x intercept. Further assays are planned to resolve these issues.

Visual fluorescence examination of MDA231/Neo and TfR cells allowed to take up rhodamine conjugated-Tf indicated a much stronger fluorescent signal for the TfR line (figure 9). This indicated that the increased TfR in this line was functional and that much more Tf was internalized by the TfR line than by the Neo line during the 2h incubation time.

One of the major properties to be ascertained concerning the high TfR expressing line was whether or not it proliferated in response to Tf to a greater degree than did the Neo line. For these assays, cells are removed from stock plates using trypsin and are plated at low density for 12 h in media containing 1% FBS. Cells are washed and serum-free media containing increasing amounts of Tf is placed in the wells. The response to Tf of the TfR line was impossible to determine since its growth in control (no added Tf) conditions was extremely rapid. When the proliferation assay was re-configured to explore this phenomenon, it was seen that the MDA231/TfR line grew at a rapid rate in 0% FBS conditions for a period of 6-8 days, whereas the MDA231/Neo line displayed no such ability (figure 10).

Of concern in observing the greater FBS-free growth rate in the TfR line was that it was acting as a Tf sink during the plating period and subsequent release of cell associated Tf was responsible for growth on following days. To determine if this was true, cells were pulse treated with ¹²⁵I-Tf and eventual uptake and release of internalized Tf was measured. This procedure revealed little difference between initial MDA231/Neo and TfR cell associated Tf (figure 11A). Released Tf was slightly higher for the TfR line, but the magnitude of the difference was not great enough to account for the increased proliferation of these cells (figure 11B).

Western blot analysis of cell and media associated Tf also revealed no significant differences between the two line's ability to internalize or secrete human Tf (figure 12). Stock cultures were washed 3 times for 1h each in alpha-MEM only. Cells were trypsinized and initially plated for 12 h in media containing 1% human serum in place of FBS. The cells were washed extensively with serum-free media, placed in serum-free media, and lysates were obtained at that time (day 1). On following days, lysates and media aliquots from like-plated and treated wells were obtained (days 2 and 3). On day 3, the MDA231/Neo cells demonstrated a large loss of viability, so only media samples were obtained. All samples were loaded onto SDS-PAGE gel at equal protein levels, separated, blotted onto nitrocellulose and analyzed

for human Tf using a polyclonal goat anti-human Tf antibody and an HRP-conjugated anti-goat IgG. HRP containing bands were detected by ECL. The results show identical or slightly less intense Tf bands in TfR cells than in Neo cells, and no detectable Tf bands in media. Differences in Tf storage or release do not explain the increased ability of MDA231/TfR to proliferate in serum-free media.

It is unknown why initial cell associated Tf was similar in these latter two experiments but obviously different when rhodamine Tf uptake was examined. Perhaps the difference in initial incubation times accounted for this.

When injected into the mammary fat pad of female nude mice, both lines were seen to form primary tumors of approximately the same size at approximately the same rate (table 1). Mice injected with the MDA231/TfR line were observed to suffer from metastases to the peritoneal mesentery, whereas those injected with MDA231/Neo cells did not. Sample photographs of these metastases are shown in figure 13. No other gross metastases were observed in any other organs. When this experiment was repeated, only four TfR injected mice were available for analysis: the remainder were dropped from the assay due to initial mis-injections or infection. The second assay also indicated that the MDA231/TfR cells formed intestinal mesentery metastases at a greater rate than did the MDA231/Neo cells (table 2). Care was taken during all injections to monitor for any mis-injections: all mice included in the final analysis were ruled out as to having received erroneous intra-peritoneally injected tumor cells.

The lungs of four mice from each group in the initial metastasis assay were analyzed for micrometastases. It was found that the lungs of mice injected with MDA231/TfR cells displayed numerous tumor cells which lacked any type of nodular organization. Only occasional lung micrometastases organized into nodules were seen in the lungs of mice receiving MDA231/Neo cells. Representative micrographs of lungs from both groups are shown in figure 13.

Discussion:

In our hands, the human MDA231 mammary adenocarcinoma cell line was seen to possess little or no transferrin receptor (TfR). For this reason, the line served as a good model for testing the ability of our TfR cDNA containing plasmid to induce the formation of TfR in transfected cell lines. This proved to be successful, with the MDA231/TfR transfected cells displaying infinitely higher (by immunofluorescence) and 6 -7 fold more (by affinity isolation and Scatchard analysis) TfR than did vector transfected controls. The TfR transfected cells were also observed to internalize more rhodamine conjugated Tf in a short time uptake assay than did the Neo transfected cells. As such, the plasmid was shown to up-regulate functional TfR in a subject cell line.

However, MDA231 cells do demonstrate a proliferative response towards transferrin (Tf; 7), and others have shown that they possess a somewhat aggressive, metastatic phenotype in nude mice (44). These two properties rendered the transfected line non-ideal for *in vivo* studies. It was to our surprise to find that the TfR transfected line displayed a greater ability to proliferate in 0% FBS conditions than did the Neo line. This observation led us to suppose that the TfR line was yet more metastatic and to test the spontaneous metastatic activity of both lines in nude mice. Here, we found that the TfR line produced greater numbers of intestinal mesentery metastases and lung micrometastases than did the Neo line.

Reports on the finding of intra-abdominal metastases are few. Meschter *et. al.* (45) have found that the MDA-MB-435 tumor cell line can form intestinal mesentery metastases in nude mice, from a thoracic mammary fat pad site. These metastases appear to localize to intestinal mesentery lymph nodes. Mechanisms of dissemination to that site and growth warrants further investigation.

These results indicate that increasing the TfR expression in a mammary adenocarcinoma cell line can affect its autocrine growth and metastatic capability. Mechanisms by which this occurs are unclear although non-iron internalization events triggered by stimulation of the TfR are suspect. We have found that certain breast cancer cell lines exhibit a sensitive, dramatic proliferative response towards

transferrin. In no or low serum conditions, the metastatic rat MTLn3 mammary adenocarcinoma cell line and the metastatic human MDA231 mammary adenocarcinoma cell line will senesce, round up, and die. The addition of 0.1 - 0.3 µg/ml of Tf will produce explosive cell growth that is similar to that obtained with the addition of 5% FBS. In contrast, other breast cancer cell lines of greater TfR expression and Tf binding capability (i.e.: MDA468, MCF7/LCC2) do not exhibit a similar proliferative response to Tf. The possibility that TfR mediated growth-promoting signal transduction events were occurring in the responsive cells is of interest. Future experiments with these transfected cell lines will explore as to whether or not these events occur.

OTHER PROJECTS:

Tf growth selection:

The development of a highly metastatic rat mammary adenocarcinoma cell subline from a low metastatic parental population by the use of an *in vitro* transferrin growth selection process was successful and outlined extensively in last year's progress report. Others involved on the paper written concerning this data requested further demonstration of intermediate metastatic capability in a subline which displayed an intermediate response to transferrin. This has been done and included in the manuscript, which is now being reviewed for submission.

TfR antisense transfection:

Previous attempts to decrease breast cancer cell TfR expression by the use of a plasmid containing an antisense TfR insert were reported on last year, where no effect on cell surface TfR protein levels were observed in transfected cells. Since then, numerous attempts have been made to construct a new TfR antisense vector. Sections of the TfR coding sequence (BamHI-KpnI or EcoRV-KpnI) were cut from pcDTR1 and ligated into another eucaryotic expression plasmid: pcDNA3 (Invitrogen, San Diego, CA). This plasmid had been cut with the same two enzymes. These sites exist in the multiple cloning region of pcDNA3 in an order which would dictate that the insert would ligate in an antisense orientation. These ligations have been unsuccessful, as none of the transformed bacteria obtained have contained a plasmid with the proper insert. A number of consultations with other investigators and Invitrogen have resulted in a number of changes in technique which are now being performed in an attempt to resolve this problem.

CONCLUSIONS:

1. Rat MTLn2 mammary adenocarcinoma cells selected for increased growth response to transferrin demonstrate increased transferrin receptor expression and an increased ability to metastasize in syngeneic fisher 344 rats (reported on last year).
2. Human A375 melanoma cells transfected with the transferrin receptor gene exhibit increased TfR expression, but do not proliferate in response to Tf. The transfected line has no increased metastatic capability in nude mice (reported on last year).
3. Human MDA231 mammary adenocarcinoma cells transfected with the TfR gene display increased TfR expression, an increased ability to proliferate in serum free conditions, and an increased metastatic capability in nude mice (this years report). An additional metastasis assay using greater numbers of mice is desired prior to submitting this for publication.
4. An antisense TfR coding plasmid does not decrease TfR expression in MCF7/LCC2 cells (reported on last year). Formation of new antisense constructs has failed to date, however, efforts are continuing in this regard.

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Appendix: Figures

Appendix: Philip Cavanaugh, Ph.D.

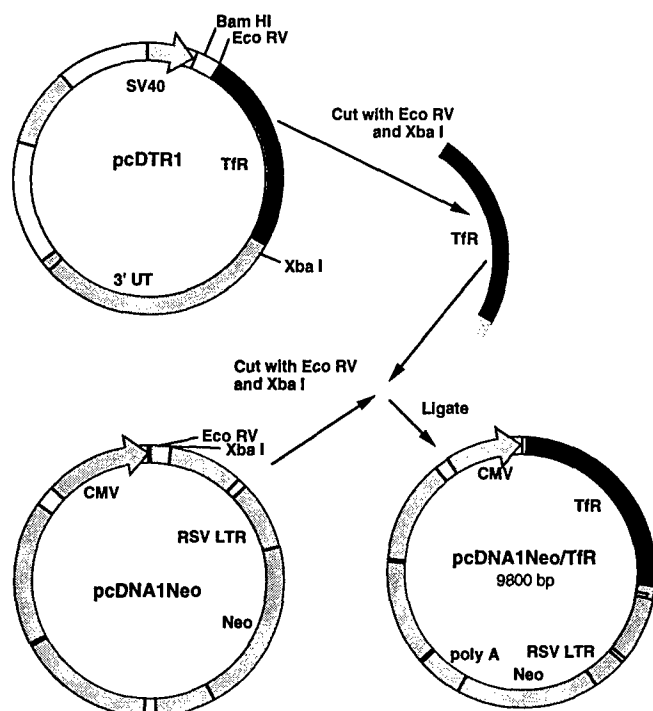


Figure 1. Schematic of the construction of the pcDNA1Neo/TfR eucaryotic expression plasmid containing the TfR cDNA. The TfR coding region was cut from pcDTR1 using EcoRV and Xba I. The coding region was ligated into pcDNA1Neo which had been cut with the same two enzymes.

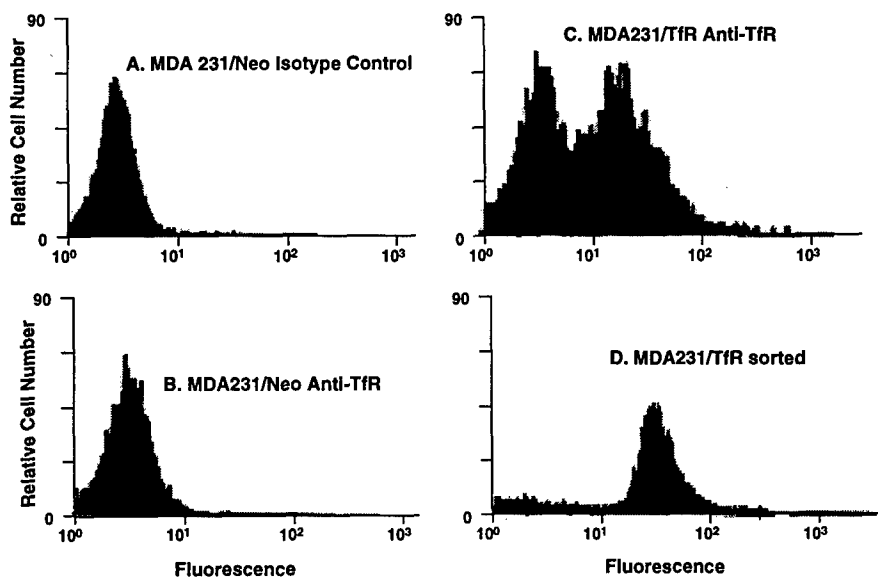


Figure 2. Initial FACS analysis and sorting of MDA231/Neo and MDA231/TfR cells. Cells were removed from plates by brief trypsinization, stained with PE-conjugated anti-human TfR or mouse IgG₁, and analyzed. A: MDA231/Neo stained with mouse IgG₁; B: the same cells stained with anti-TfR. C: MDA231/TfR stained with anti-TfR. D: FACS analysis of the population of cells obtained when the top 5% of fluorescing cells were sorted from C. MDA231/TfR cells when stained with mouse IgG₁ produced a signal similar to that shown in panel A.

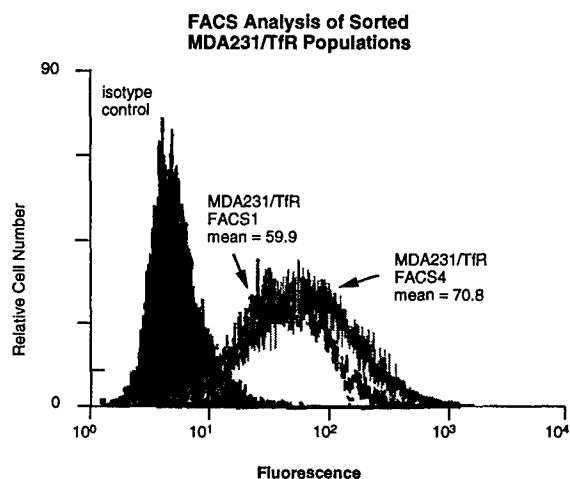


Figure 3. FACS analysis of MDA231/TfR cells from the first and fourth sequential FACS sorts for high TfR expressors. Cells were stained with PE-conjugated anti-TfR, sorted for high expression, re-cultured, and sorted again. This process was repeated four times. Cultured cells from the first and final sort were re-treated and re-analyzed.

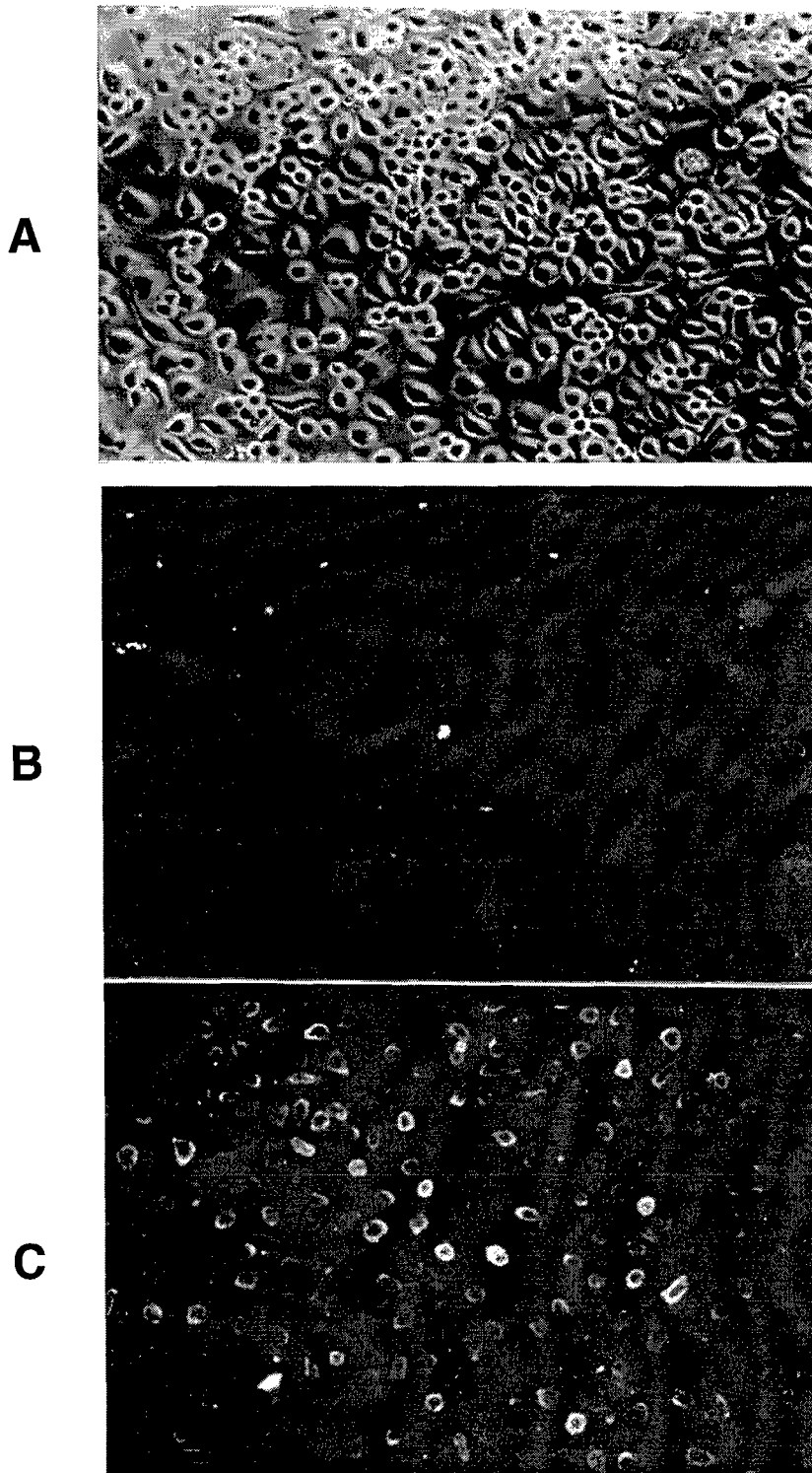


Figure 4. Micrographs of MDA231/Neo and MDA231/TfR cells stained with PE-conjugated anti-TfR. A: MDA231/Neo (visible); B; fluorescent view of A; C: MDA231/TfR from the fourth FACS sort (fluorescent).

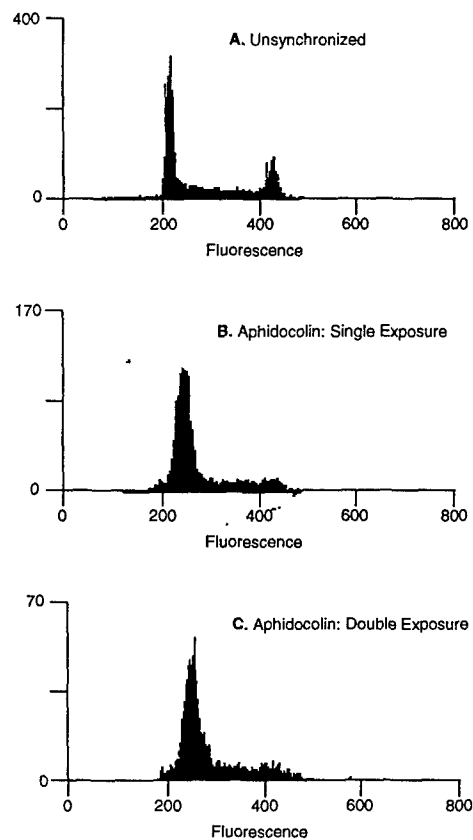


Figure 5. Synchronization of MDA231/TfR cells with aphidocolin. Cells at 50% confluence were treated with 3 μ M aphidocolin for 12 h and analyzed (panel B) or placed back into aphidocolin-free media for 12 h, re-treated with aphidocolin for 12 h and analyzed (panel C.) Panel A shows the results for unsynchronized cells. All cells were stained with propidium iodide prior to analysis.

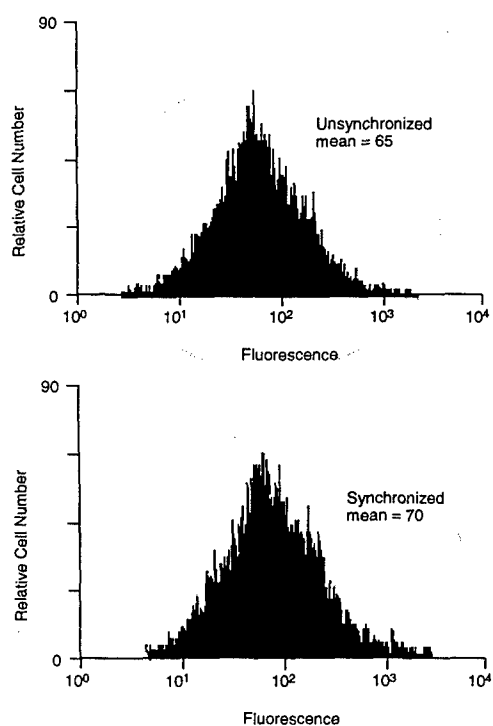


Figure 6. Results of analysis of cell surface TfR on unsynchronized and aphidocolin synchronized (panel C from figure 5) MDA231/TfR cells. Cells were removed from culture plates by brief trypsinization, stained at 4°C with PE-conjugated anti human TfR, washed, and analyzed.

MDA231/Neo, MDA231/TfR: TfR Quantitation by Affinity Isolation

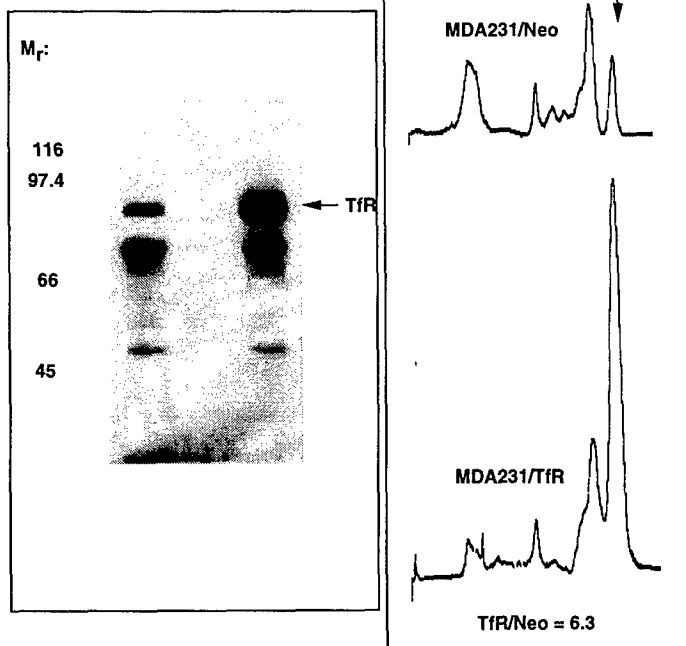


Figure 7. Results of cell surface TfR measurement on MDA231/Neo and MDA231/TfR cells by affinity isolation of TfR. Cells were surface biotinylated at 4°C, lysed, and equal cell equivalents of each lysate were incubated with a excess of Tf-agarose. Agarose bound material was separated by SDS-PAGE, blotted onto PVDF, incubated with streptavidin-HRP, and HRP containing bands were detected by ECL. TfR bands were quantitated with by scanning densitometry (right panel).

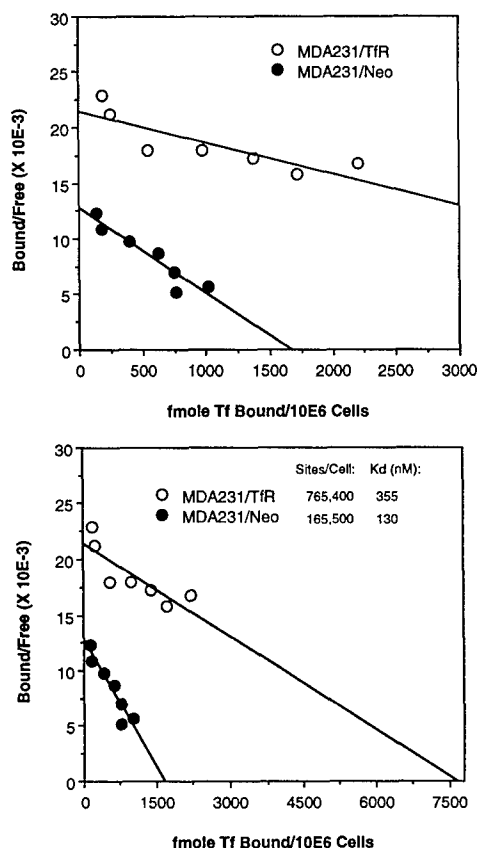


Figure 8. Measurement of cell surface TfR on MDA231/Neo and MDA231/TfR cells by Scatchard analysis. Cells were grown in 12 well plates to 60-70% confluence. Cells were incubated at 4°C with increasing levels of ^{125}I -Tf with or without an excess of cold Tf for 2h. Cells were washed and lysed. Lysate and unbound counts were determined and plotted. Points represent data from triplicate wells.

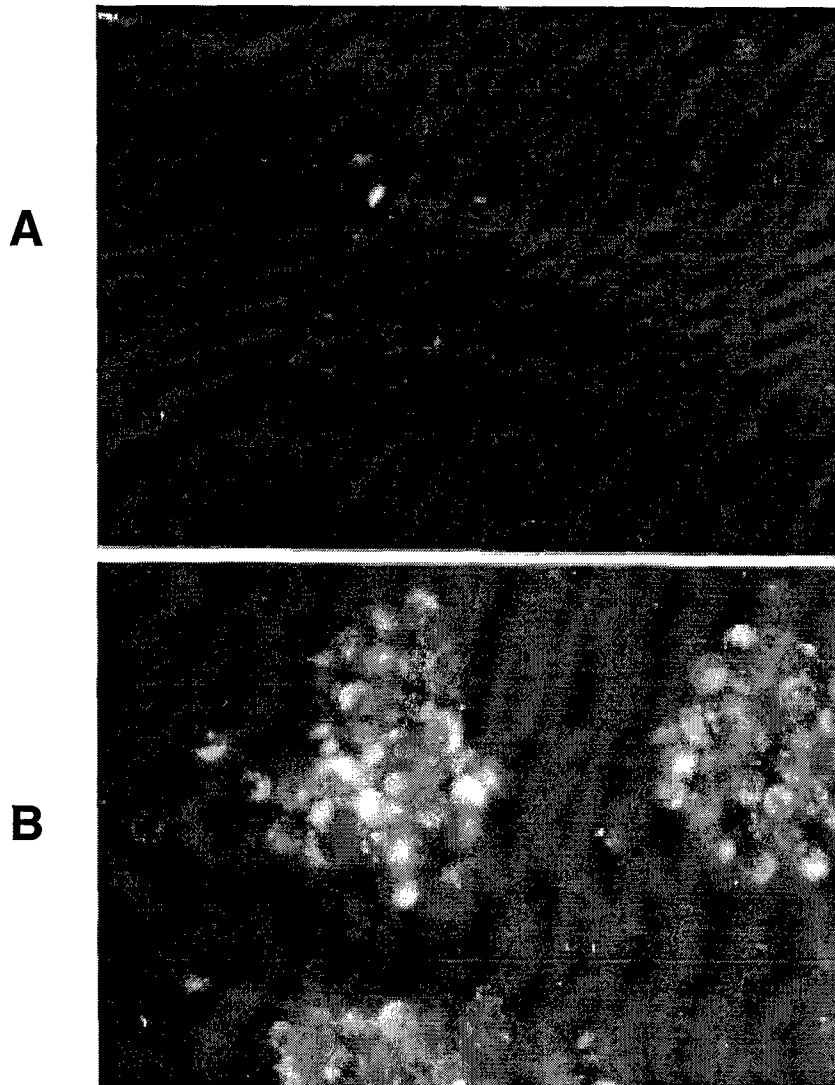


Figure 9. Fluorescent micrographs of MDA231/Neo and MDA231/TfR cells allowed to take up rhodamine-conjugated transferrin. A: MDA231/Neo cells; B: MDA231/TfR cells. Cells were of approximately equal density on the slides when treated.

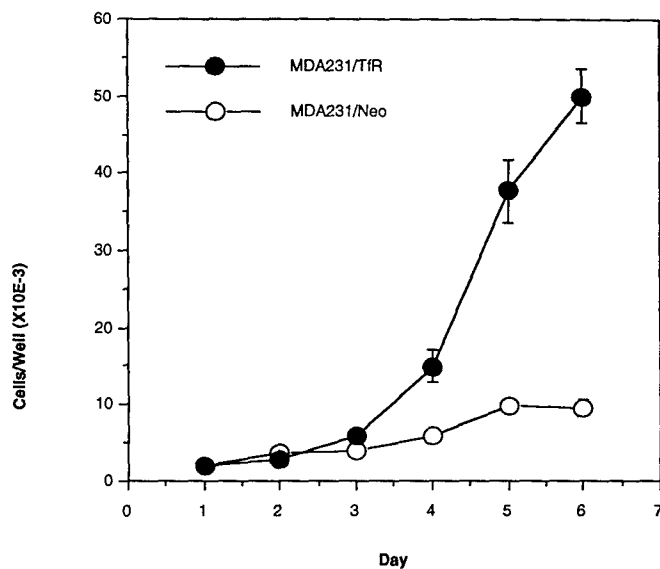


Figure 10. Proliferation of MDA231/Neo and MDA231/TfR cells in 0% FBS containing media. Cells were plated at 2,000 cells/well in 96 well plates in media containing 1% FBS. 12 h later, cells were washed and placed in FBS-free media. On ensuing days, cells in select wells were trypsinized and counted using a Coulter Counter. Points are mean \pm SD from quadruplicate wells

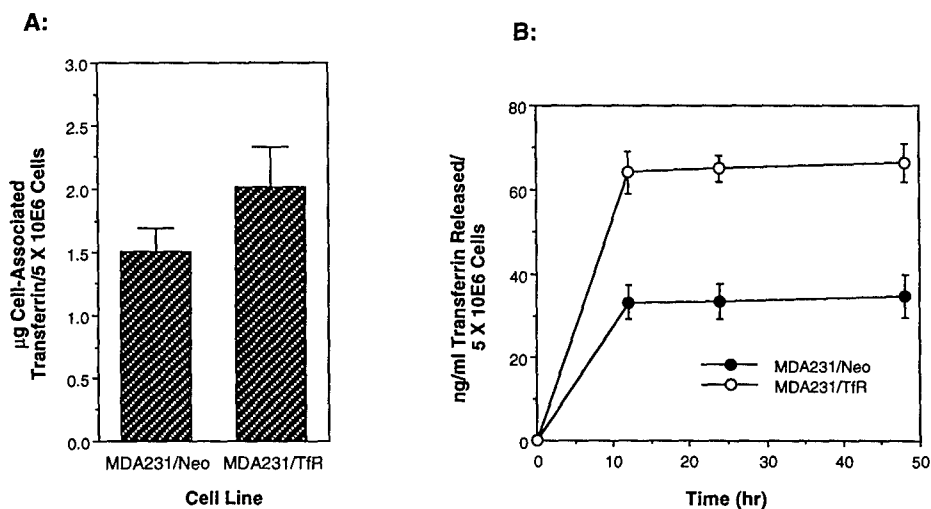


Figure 11. Uptake and release of ^{125}I -Tf by MDA231/Neo and MDA231/TfR cells. Cells were incubated in serum free conditions for 8 h with 0.5 $\mu\text{g/ml}$ ^{125}I -Tf. Cells were washed and cell bound radioactivity determined in three wells of each (panel A). Radioactivity in aliquots of media from other wells was determined at various subsequent time points (panel B).

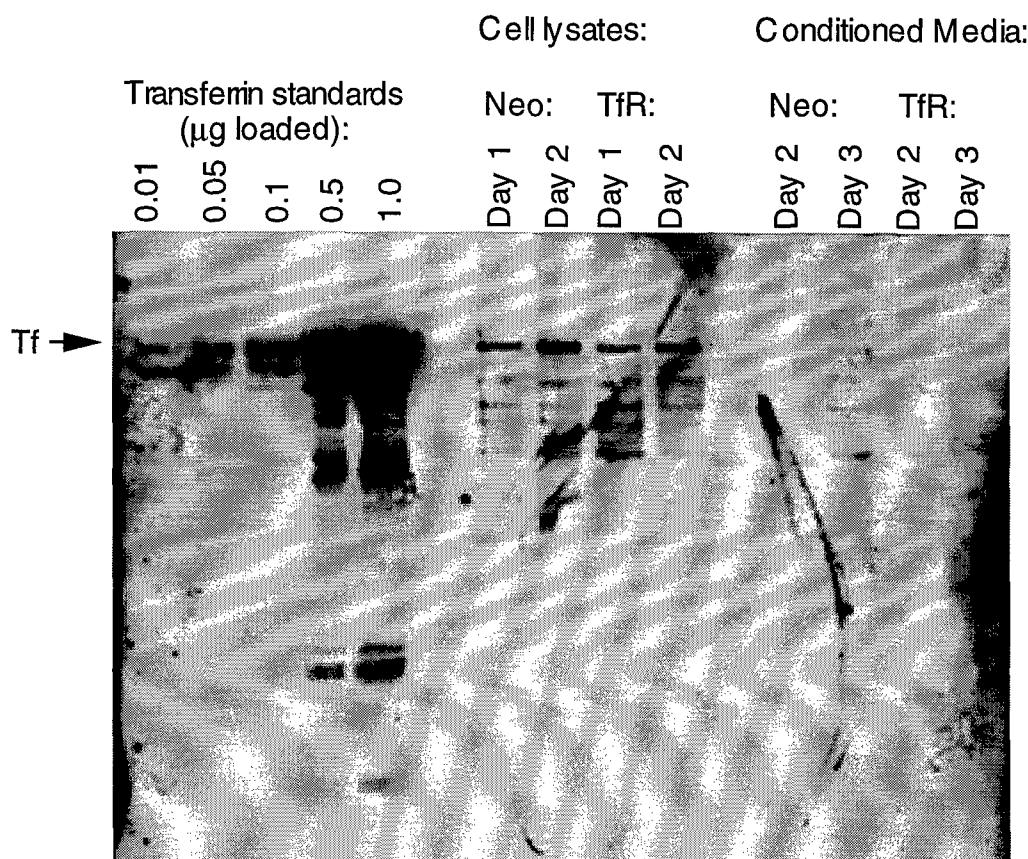


Figure 12. Western blot analysis of transferrin uptake and release in starved MDA231/Neo and MDA231/TfR cells. Washed cells were allowed to plate in media plus 1% human serum for 12 h. Cells were washed and day 1 lysates obtained. 24 h later, day 2 lysates and media were harvested. On day 3, MDA231/Neo cells were nearly dead, so media only was analyzed. Thirty μg lysate protein was loaded into lysate lanes; 100 μg of media protein was loaded into media lanes. Lysates and media were separated by SDS-PAGE, blotted onto nitrocellulose, incubated with goat anti-human Tf, then with HRP-anti-goat IgG. HRP containing bands were detected by ECL.

MDA231/TfR:

Mouse	1° Tumor Dimensions (cm):	Metastases:
1	0.5 X 0.2 X 0.75	ascites/mesentery
2	0.75 X 0.75 X 0.5	ascites/mesentery
3	none	none
4	1.3 X 1.0 X 0.75	ascites/mesentery
5	2.0 X 1.7 X 0.5	ascites/mesentery

MDA231/Neo:

Mouse	1° Tumor Dimensions (cm):	Metastases:
1	2.0 X 2.0 X 0.3	none
2	2.0 X 1.0 X 0.5 + 1.5 X X 0.8 X 0.8	none
3	0.8 X 1.0 X 0.5	none
4	0.5 X 0.3 X 0.3	none
5	1.0 X 0.75 X 0.3	none

Table 1. Results of the first spontaneous metastasis assay. Female nude mice were injected into the left thoracic mammary fat pad with 1×10^5 cells. Four weeks later, animals were sacrificed and examined for gross metastases.

MDA231/TfR:

Mouse	1° Tumor Dimensions (cm):	Metastases:
1	none	none
2	1.5 X 1.5 X 0.5	none
3	1.5 X 1.2 X 1.0	ascites/mesentery
4	2.0 X 1.2 X 1.0	ascites/mesentery
5	1.5 X 1.5 X 1.1	none

MDA231/Neo:

Mouse	1° Tumor Dimensions (cm):	Metastases:
1	2.0 X 2.0 X 1.0	none
2	1.8 X 1.5 X 1.0	ascites/mesentery
3	1.8 X 1.2 X 1.0	none
4	1.5 X 1.5 X 1.0	none
5	2.0 X 2.0 X 1.5	none
6	2.2 X 2.0 X 1.3	none

Table 2. Results of the second spontaneous metastasis assay. Female nude mice were injected into the left thoracic mammary fat pad with 1×10^5 cells. Four weeks later, animals were sacrificed and examined for gross metastases.

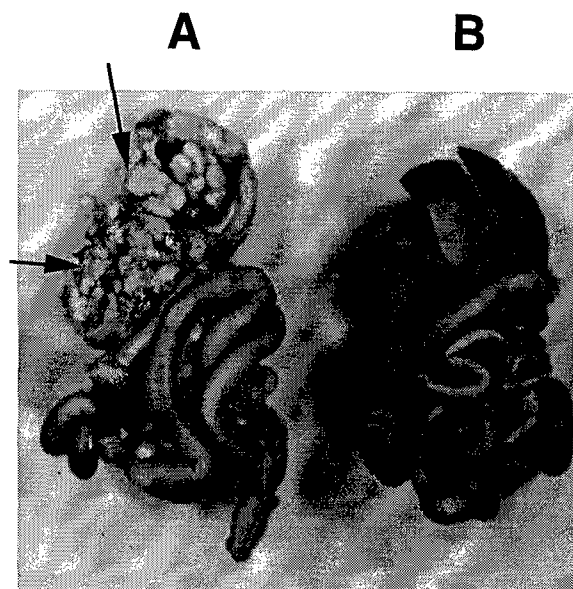


Figure 13. Representative photographs of intraperitoneal organ blocks from nude mice injected with MDA231/TfR cells (A) or MDA231/Neo cells (B). Metastases in A are indicated by the arrows.

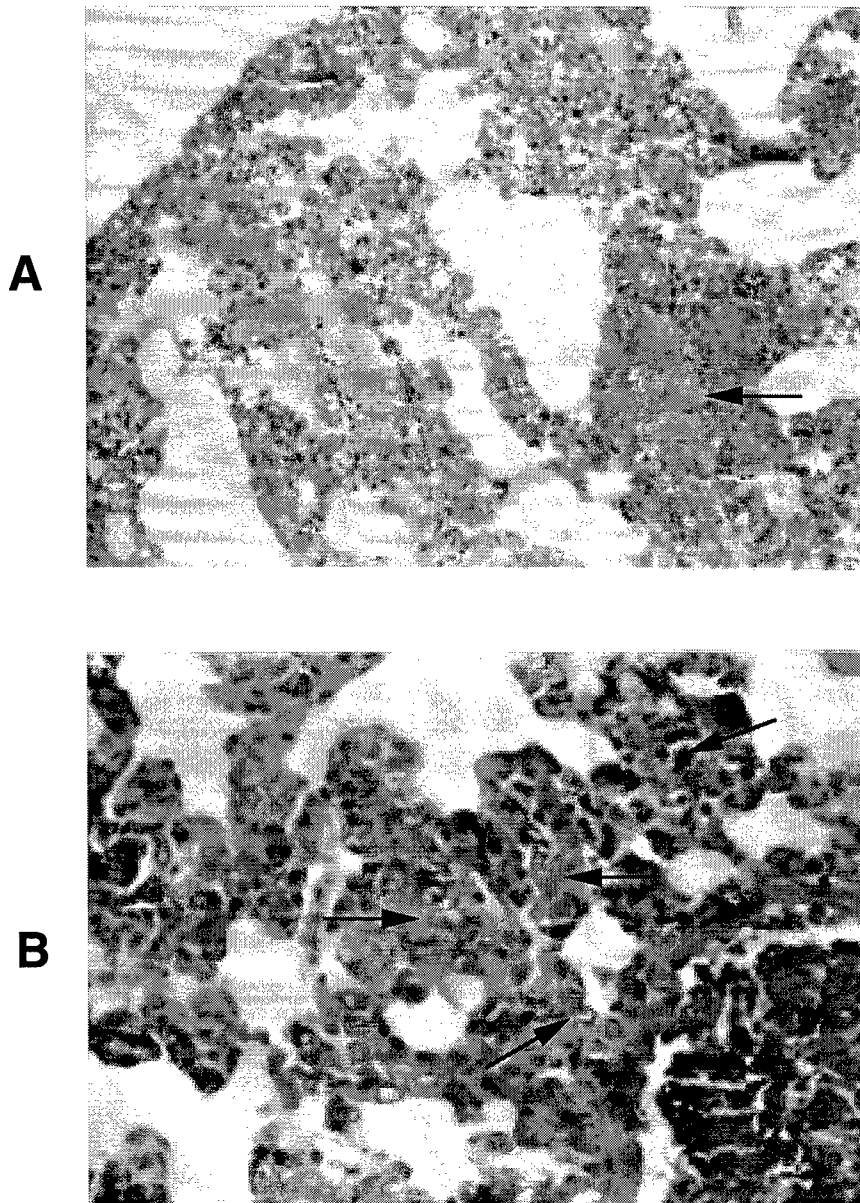


Figure 14. Representative lung sections from nude mice injected with MDA231/Neo cells (A) or MDA231/TfR cells (B). A rare nodular lung metastasis is indicated in A (arrow) and multiple dispersed tumor cells lacking a nodular appearance are indicated in B (arrows).

Biographical Sketch (Bibliography)

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Publications:

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Personnel receiving pay from this grant:

Philip G. Cavanaugh



Rec'd
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